



Patent Application
Docket No. USF-167XC1
Serial No. 10/815,388

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner : Allison M. Ford
Art Unit : 1651
Applicants : Pablo Caviedes, Raul Caviedes, Thomas B. Freeman, Juan A. Asenjo, Barbara A. Andrews, Dario Sepúlveda, Christian Arriagada, Julio Salazar Rivera
Serial No. : 10/815,388
Filed : March 31, 2004
For : Materials and Methods for Regulating Process Formation in Cell Culture

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF PABLO CAVIEDES, M.D., UNDER 37 C.F.R. §1.132

Sir:

I, Pablo Caviedes, M.D., Ph.D., hereby declare:

THAT, I am a professor in the Program of Molecular and Clinical Pharmacology, ICBM, Faculty of Medicine at the University of Chile;

THAT, my *curriculum vitae* is attached hereto as Exhibit A;

THAT, through my years of research, I have kept up to date on the technical literature and maintained contact with experts in the field by participating in professional meetings and seminars, and by direct personal contact. As a result, I am familiar with the general level of skill of those working in the fields of cell culture and neurobiology, and particularly as they relate to neural cell transplant therapy for treatment of neurodegenerative disorders;

THAT, I am a co-inventor of the technology described and claimed in patent application Serial No. 10/815,388 (hereinafter the '388 application);

THAT, I have read and understood the specification and claims of the '388 application, the Office Action dated April 5, 2006, and the references cited in the Office Action;

AND, being thus duly qualified, do further declare:

1. Claims 18, 21-26, 30, 31, and 35-39 have been rejected under 35 U.S.C. §103(a) as being obvious over Takazawa *et al.* (U.S. Patent No. 5,219,752) in view of Studer *et al.* (Published International Application No. WO 00/05343) and further in view of Boss *et al.* (U.S. Patent No. 5,411,883).
2. The cell culture of the invention would not have been obvious to a person of ordinary skill in the art at the time the invention was made, based on the references cited in the Office Action. Claims 18 and 39 of the '388 application recite that the cell culture comprises process-forming neuronal cells of the central nervous system and has a calcium concentration of 100 μ M or less. The empirical data in columns 17-22 of the Takizawa *et al.* patent indicate that the aggregation of fetal kidney cells essentially occurs when a threshold calcium concentration is reached. In contrast, we have found that process-forming neuronal cells of the central nervous system will aggregate when cultured at a calcium concentration of 100 μ M or less. The Takazawa *et al.* patent proposes that various adherent animal cells can be cultured using the method disclosed therein, including the 500+ cells tabulated in columns 5-12. These cells represent a very diverse variety of tissues, *e.g.*, bat lung cells, goldfish fin cells, goose sternum cells, human bone marrow cells, human breast cells, human pancreatic cells, mosquito larval cells, moth ovarian cells, snail embryonic cells, viper spleen cells, *etc.* However, the only cells described in the Takazawa *et al.* patent as actually being cultured with the disclosed method are kidney cells, *i.e.*, 293 cells (human fetal kidney) and BHK 229 cells (hamster kidney). Absent supporting empirical data, such as that provided in the '388 application, one of ordinary skill in the art would not have a reasonable expectation of success in creating a cell culture comprising neuronal cells of the CNS that cluster into aggregates, as recited in claims 18 and 39.
3. Assuming for the sake of argument that the references cited in the Office Action support a reasonable expectation of success in producing the cell culture of the invention, the results we have obtained using neuronal cells of the cell culture were significantly better than could have been expected. We implanted suspensions of the RCSN-3 cell line (a rat substantia nigra cell line) into the striatum of rats previously lesioned in the nigrostriatal pathway with 6-hydroxydopamine (6-OHDA).

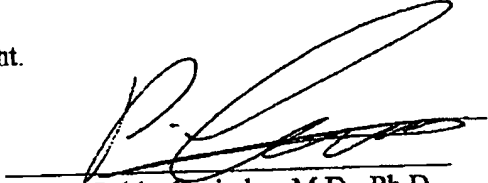
This toxin-induced animal model is well known and accepted for simulating the motor deficits occurring in Parkinson's disease, and considered appropriate for experiments on morphological and behavioral recovery after partial lesioning of the nigrostriatal DA system (Iancu R. *et al.*, *Behavioral Brain Research*, 2005, 162:1-10; Nishimura, F. *et al.*, *Stem Cells*, 2003, 21:171-180; Bjorklund L.M. *et al.*, *PNAS*, 2002, 99(4):2344-2349; Shingo T. *et al.*, *Journal of Neuroscience Research*, 2002, 69:946-954; Mukhida K. *et al.*, *Journal of Neuroscience*, 2001, 21(10):3521-3530; Sawamoto K. *et al.*, *PNAS*, 2001, 98(11):6423-6428; Dabben-Sali F. *et al.*, *FASEB J.*, 2001, 15:164-170; Dunah A.W. *et al.*, *Molecular Pharmacology*, 2000, 57:342-352; Bilang-Bleuel A. *et al.*, *PNAS USA*, 1997, 94:8818-8823). Submitted herewith as Exhibit B is a book chapter entitled "An Immortalized Neuronal Cell Line Derived from the Substantia Nigra of an Adult Rat: Application to Cell Transplant Therapy", which describes the implantation of the RCSN-3 cell line using conventional culture methods. As indicated in Example 1 of the '388 application, the RCSN-3 cell line was established from a primary culture of the striatum of Fisher 344 rats, and exposed to media conditioned with the UCHT1 cell line (Caviedes R. and Stanbury J.B., *Endocrinology*, 1976, 99:549-554). RCSN-3 cells retain the morphology of the neuronal phenotype (Cardenas A.M. *et al.*, *Neuroreport*, 1999, 10(2):363-369). Submitted herewith as Exhibit C is a graph showing results from implantation of the RCSN-3 cell line using the cell culture of the invention. Gradual behavioral recovery (reduction of apomorphine-induced rotation scores) was observed in transplanted animals. As shown in Figure 6 of Exhibit B, rats implanted with conventionally cultured RCSN-3 cells showed a steady decrease in rotations, leveling off at 75% of the initial rotation values after approximately 12-16 weeks post-implant. In contrast, when obtained from the cell culture of the invention described in Example 1 of the '388 application, RCSN-3 cells reached a plateau significantly sooner, at approximately 6 weeks post-implant. The comparative data demonstrate that the cell culture of the invention is particularly advantageous for cell transplantation. This improvement in apomorphine-induced circling behavior would be considered significant and unexpected by those of ordinary skill in the art.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed:


Pablo Caviedes, M.D., Ph.D.

Date:

October 4th, 2006

EXHIBIT A

CURRICULUM VITAE



NAME : PABLO ANDRES CAVIEDES.
BIRTHDATE : DECEMBER 28, 1960.
COUNTRY OF CITIZENSHIP : CHILE.
MAILING ADDRESS : PROGRAM OF MOLECULAR & CLINICAL PHARMACOLOGY.
ICBM, FACULTY OF MEDICINE, UNIV. OF CHILE
CLASIFICADOR 7 – INDEPENDENCIA. INDEPENDENCIA 1027
SANTIAGO, CHILE
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PRESENT POSITION : - ASSOCIATE PROFESSOR, ICBM. FACULTY OF MEDICINE,
UNIVERSITY OF CHILE.
MARITAL STATUS : MARRIED, THREE CHILDREN.
LANGUAGES : SPANISH, ENGLISH, FRENCH.

EDUCATION

- 1.- High school graduate: The Grange School, Santiago, Chile. December 14th, 1978.
 - 2.- Medical Doctor degree: Faculty of Medicine, University of Chile, Santiago, Chile. January 3rd, 1986.
 - 3.- Ph.D. in Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile. January, 1998.
- Thesis: Alterations of electrical membrane properties in sensory neurons from human trisomy 21 and murine trisomy 16 subjects.

POSITIONS

- August, 1982- April, 1985 : Pre graduate assistant, ad honorem.
Dept. of Physiology & Biophysics,
Faculty of Medicine, University of Chile.
- March, 1984- Dec., 1984 : Pre graduate assistant, ad honorem.
Public Health Institute.
Faculty of Medicine, University of Chile.
- May, 1985- Dec., 1985 : Pre graduate Faculty appointment.
Dept. of Physiology & Biophysics.
Faculty of Medicine, University of Chile.
- January, 1986- Sept., 1986 : Post doctoral research assistant,
Dept. of Physiology & Biophysics.
Faculty of Medicine, University of Chile.
- Sept., 1986- Dec., 1989 : Visiting Fellow, Fogarty International
Center, Laboratory of Neurosciences, National
Institute on Aging, NIH.
- Sept. 1989- Sept. 1990 : Cystic Fibrosis Foundation Fellowship.

- January, 1990- March, 1991 : Visiting Associate, Fogarty International Center, Laboratory of Cell Biology & Genetics, National Institute on Diabetes, Digestive and Kidney Diseases, NIH.
- April 1, 1991- 2000 : - Assistant Professor.
Centro de Estudios Científicos de Santiago.
Santiago, Chile.
- February 1st, 1992- May, 1997 : - Faculty position, Assistant Professor Level,
Dept. of Physiology & Biophysics
Faculty of Medicine, University of Chile.
- May, 1997 - present : - Faculty position, Assistant Professor Level,
Institute for Biomedical Sciences (ICBM)
Faculty of Medicine, University of Chile.
- August, 2000 – March 2002 : - Senior Researcher
Sansum Medical Research Institute
Santa Barbara, CA. USA
- September, 2003 – present : - Associate professor. Institute for Biomedical Sciences (ICBM)
Faculty of Medicine, University of Chile.
- July, 2005 – present : - Deputy Director, Program of Molecular & Clinical Pharmacology.
ICBM, Faculty of Medicine, Univ. of Chile
- January, 2006 – present : - Director, Center for Clinical Research & Pharmacological Studies (CICEF),
Faculty of Medicine, University of Chile.

EXAMINATIONS

- 1977: First Certificate in English, Local Examinations Syndicate,
University of Cambridge, England. Grade A Award.
- 1978: Certificate of Proficiency in English, Local Examinations Syndicate,
University of Cambridge, England. Grade C Award.
- 2004: TOEIC, CORFO (Chile). Level A (Maximal score, Professional).

SOCIETIES, HONORS

- PhD Program Fellowship, Conicyt (Chile), from April 1991 to June 1994.
- Member of the Society for Neuroscience since April, 1991.
- Member of the International Advisory Board of the Cell Transplant Society since July, 1991.
- Member of the New York Academy of Sciences since November, 1991.
- Member of the Biology Society of Chile since January, 1994.
- Member of the Scientific Committee of the Alzheimer Corporation - Chile, since May, 1993.
- Member, International Brain Research Organization (IBRO), since December 1994.
- Member, Board of Directors, Chilean Society for Physiological Sciences. March 1998.
- Member, Organizing Committee, Joint Meeting Chile-UK Physiological Societies Meeting. Pucón, Chile,

November 13-16, 1999.

- Grant proposal referee, Fondecyt (National Fund for the Development of Science & Technology), Chile, since 1991.
- Grant proposal referee, Foncyt (National Agency for the Promotion of Science & Technology), Argentina, since May 1999.
- Member, Organizing Committee, International Meeting on Neurodegeneration, March 16-18, 1, 2001.
- Regional Director, Neurotoxicity Society, since June 2001.
- Executive secretary, Organizing Committee, Neurotoxicity Meeting: mechanisms for neurodegenerative disorders- Alzheimer, ALS and Parkinson's disease. La Serena, Chile, April 25-27, 2003.
- Director, International Workshop: "Clinical Trials: Present and future under the new legislation". September 28, 2006. Santiago, Chile.

COURSES

- April 23-26, 1984 : 'Techniques in ionic channel recordings'. Faculty of Science, University of Chile (32 hrs.)
- March-December, 1984 : 'Major Clinical Problems'. Dept. of Medicine, Faculty of Medicine, University of Chile (42 hrs.)
- October 7-Nov. 18, 1985 : 'Growth and Development of the Normal Child'. FUDOC, Ministry of Health, Stgo., Chile (14 hrs).
- March, 1986-August, 1986 : 'Cell Physiology, Dept. of Physiology & Biophysics, Faculty of Medicine, University of Chile.
- April 21-26, 1986 : Assistant in the course 'Techniques in Ionic Channel Recordings'. Centro de Estudios Científicos de Santiago; Faculty of Science, University of Chile (44 hrs.).
- April 4, 1987 : Using animals in Intramural Research: Guidelines for Investigators. NIH Training Center (4 hrs.).

RESEARCH EXPERIENCE

- 1982- 1985: Dept. of Physiology & Biophysics, Faculty of Medicine, University of Chile; under the supervision of Professor Enrique Jaimovich. Investigation of the electrophysiology of several tissue culture cell lines, using intracellular microelectrode and patch clamp; also acquiring experience in various tissue culture techniques (basic tissue culture, transformation, cell fusion, cloning) with different cell systems (neuronal, neuroendocrine, myocardium, endothelium, etc.) under the guidance of Professor Raúl Caviedes.
- 1984- 1985: Institute of Public Health, Faculty of Medicine, University of Chile; under supervision of Professor Dr. María Inés Romero. Investigation of psychological disorders in adolescence.
- Sept., 1986-
Dec., 1989: Visiting Fellow, Fogarty International Center, Laboratory of Neurosciences, National Institute on Aging, NIH; under the sponsorship of Dr. Stanley I. Rapoport. Study of ionic membrane currents in cultured dorsal root ganglia neurons from normal and trisomy 21 (Down syndrome) human fetuses, trisomy 16 and trisomy 19 fetal mice, using the patch clamp technique for whole cell and single channel recording. Study of the effect of Nerve Growth Factor in the electrical membrane properties of dorsal root ganglia neurons from normal and trisomy 21 human fetal tissue. Further experience has been gained in the study of electrical responses of excitable cell membranes, namely action potential, whole cell and single channel currents, using intracellular microelectrode and patch clamp recording techniques. This has required

learning the operation, design and construction of the electronic equipment needed for adequate recording of cellular electrical responses, based in multiple configurations using operational amplifiers. The experience is complemented with thorough knowledge in the use of personal computers applied to electrophysiological work, involving the handling of data acquisition hardware and software systems, and knowledge of microcomputer programming languages (i.e. FORTRAN, BASIC, C) which provides the ability to produce personal computer programs for data analysis when necessary.

Jan., 1990-

March, 1991: Visiting Associate, Fogarty International Center, Laboratory of Cell Biology & Genetics, National Institute on Diabetes, Digestive and Kidney Diseases, NIH; and Cardiorrenal Drug Division, FDA: Study of electrical membrane properties, voltage and agonist- activated membrane currents in endothelial cells in culture, and their modulation by drugs.

March, 1991-

present: Laboratorio de Cultivo de Tejidos, Facultad de Medicina, U. de Chile. Research lines continuing with the work initiated at NIH (see Projects). Collaboration with Dr. Enrique Jaimovich in projects funded by the Muscular Dystrophy Association and Fondecyt, consistent in the electrophysiological study of muscle cell lines. Also, establishment of neuronal cell lines from the trisomy 16 mouse model, and their characterization using morphological, cytogenetical, pharmacological and electrophysiological techniques, in collaboration with Dr. Stanley Rapoport, LNS/NIA/NIH. We are also studying the expression of Ca^{2+} channels in our neuronal cell lines in collaboration with Dr. Takeshi Shimahara, Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, at Gif-sur-Yvette in France. Finally, we are studying a rat *substantia nigra* cell line as a model of cell pathophysiology in Parkinson's disease, and its use in cell transplant therapy.

FUNDED PROJECTS

TITLE	FUNDING AGENCY
Alterations of electrophysiological properties in trisomy 16 mice, a model for human trisomy 21(Down's syndrome). Principal Investigator.	Fogarty International Center, NIH. Bethesda, MD. April, 1991. (US \$14.910). 1 year.
Alterations of cellular properties of cultured endothelial cells in response to varying external protein content. Principal Investigator.	Fogarty International Center, NIH. Bethesda, MD. April, 1991. (US \$14.910). 1 year.
Human cell lines from normal and dystrophic skeletal muscle. Co-investigator	Muscular Dystrophy Association, USA. 1990. (US\$98.000). 3 years.
Study of secretion and electrophysiology of umbilical vein endothelial cells from normal and hypertensive patients. Co-investigator.	D.T.I., Univ. Of Chile. November, 1991 (US\$ 8.000). 2 years.
Alterations of electrical membrane properties of excitable tissue in culture derived from the trisomy 16 mouse, a model for Down syndrome. Principal Investigator.	Internal Project, Faculty of Medicine, Univ. of Chile, 1993. (US \$1.000). 1 year.
Mechanisms of regulation of intracellular Calcium in cultured skeletal muscle cells. Co-investigator.	Fondecyt, Chile. 1993 N° 1931089 (US\$100.000). 3 years
Characterization of human cell lines from normal and dystrophic skeletal muscle. Co-investigator	Muscular Distrophy Association, E.E.U.U.. E.E.U.U. 1994. (US\$110.000). 3 years.
A cellular model for Down syndrome to evaluate alterations in electrical membrane properties in neurons: Establishment and characterization of immortal cell lines from neuronal tissue. Principal Investigator	Fondecyt, Chile, 1995. N° 1950485. (US\$110.000). 3 years.

Characterization of voltage sensitive Calcium channels and intracellular Calcium release in a rat cerebellar cell line. Co-investigator

Dirección Invest., Univ. of Valparaíso, 1994 (US\$15.000). 3 years.

Calcium regulation in nerve and muscle cells. Co-investigator

European Economic Community Grant Intl. Grant (France, UK and Chile). 1995. US (US\$60.000). 3 years.

Immortal cell lines derived from the nervous system of trisomy 16 mice, and animal model for human trisomy 21 (Down syndrome).
Principal Investigator.

Fondecyt, Chile, 1998
N° 1980906.
(US\$200.000)
3 years.

Immortal cell lines derived from the nervous system of trisomy 16 mice, and animal model for human trisomy 21 (Down syndrome).
Principal Investigator, complementary to Fondecyt N° 1980906.

Fondecyt, Chile, 1998
N° 7980058.
(US\$10.000)
3 years

Excitation-transcription mechanisms in skeletal muscle cells.
Collaborator

Fondecyt, Chile, 1998
N° 9890010
(US\$ 360.000). 3 years

Ca²⁺ signals in neuronal cell lines derived from normal and trisomy 16 fetal mice
Co investigator

Dirección Invest.,
Univ. Valparaíso
N° 0398. 1998
(US\$10.000 per year). 2 years

Evaluation of immortalized cell lines for cell transplant therapy MPTP Parkinsonian monkeys. Principal Investigator, Chile

Programa de cooperación científica con Iberoamérica. Ministry of Education, Spain. (US\$8000, 1st year), 2001

Center for Cell Transplant Therapy

Academic Division, Clínica Las Condes, Santiago, Chile (US\$10.000). 2001

Neurotoxicity mechanisms and neuroprotection in cellular and animal models of Parkinson's disease. Coinvestigator

ECOS/Conicyt 2001 (3 years, 6000 €/year).

Optimization of cell proliferation and differentiation in vitro: Application to cell transplant therapy. Principal Investigator

National Priority Areas grant, DID, Univ. of Chile. (US\$23.000). 2002-2003

Studies of toxicity mechanisms of dopamine derived o-quinones derivadas and possible polymorphisms of DT- -diaphorase and GST in Chilean Parkinson's patients. Main coinvestigator	Fondecyt Chile, 2002. N° 1020672. (US\$195.000). 4 years
Molecular and cellular damage mechanisms by gene overexpression in neuronal cell lines: Pathology and therapy in Down syndrome and Alzheimer's disease. Principal Investigator	DID, Univ. of Chile. (US\$3.500). 2002
Establishment of a human substantia nigra cell line for use in cell transplant Parkinson's therapy of Parkinson's disease. Principal Investigator	The Coalition to Cure Parkinson's Disease. Tampa, FL, USA. (US\$ 6.000) 2002.
Cell models of human Down syndrome: Role of gene overexpression in neuronal dysfunction of immortalized cells from the nervous system of trisomy 16 mice. Principal Investigator	Fondation Jérôme Lejeune, Paris, France. (45.000 €). 2003, 3 years.
Gene dosage effects in cholinergic and glutamatergic dysfunction of immortalized neuronal cells derived from the trisomy 16 mouse, an animal model of human Down syndrome. Principal Investigator.	Fondecyt 2004 N° 1040862 (US\$210.000). 3 years.
Role of Down syndrome related genes in Intracellular Calcium alterations in a neuronal Cell line derived from the cerebral cortex of a Trisomy 16 mouse, an animal model for Down Syndrome. Principal Investigator.	CNRS/Conicyt Exchange Program (2004 – 2006)
Chemical, pharmacological and toxicological studies of the <i>Haplopappus multifolius</i> and <i>H. taeda</i> . Coinvestigator	Agricultural Innovation Fund. Ministry of Agriculture, Chile. (2006). 1 year.
Studies on the molecular mechanisms of Parkinson's disease: Neuroprotective role of DT-diaphorase and GST M2-2 in cell culture and coexistence of DT-diaphorase and alfa-synuclein with Parkinson's disease. Coinvestigator	Fondecyt 2006 (Chile), N° 1061083. 4 years.
Purification and Characterization of a Transformation Factor Secreted by a Rat Thyroid Tumor Cell Line. Main Coinvestigator	FHTC Matching Funds Research Program. Tampa, Florida. EEUU 2006- 2007 (US\$ 155.000)

INVITED CONFERENCES, LECTURES (1996 - present)

- Invited speaker, September, 1996. Mount Sinai Medical Center, University of Wisconsin, Milwaukee, WI. USA.
- Invited speaker, September 1996, Dept. of Physiology, Texas Tech University Health Science Center, Lubbock, TX, USA..
- Invited speaker. September 1996. Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, Gif-sur-Yvette, France.
- Invited speaker, October, 1996. A.I. Virtanen Institute, University of Kuopio, Kuopio, Finland
- Invited speaker, October 1996. Faculty of Medicine, University of Oulu. Oulu, Finland
- Invited speaker, October 1997. Faculty of Medicine, University of Leuven, Belgium.
- Invited speaker, September 1998, Dept. of Pharmacology, School of Pharmacy, Texas Tech University Health Science Center, Amarillo, TX, USA..
- Invited speaker, September 1999, Dept. of Pharmacology, School of Pharmacy, Texas Tech University Health Science Center, Amarillo, TX, USA..
- Invited speaker, February 2000. Samsom Medical Research Institute, Santa Barbara, California. USA..
- Invited speaker. Symposium, Congress of the Latinamerican Soc. of Physiology. September, 2000. Cancun. Mexico.
- Invited speaker. October 2000. Symposium, Solvay Pharmaceuticals (Holland), on mechanisms in Parkinson's disease. Como, Italy.
- Invited speaker. Symposium "Advances in the treatment of Parkinson's disease", Congress of the Chilean Society of Neurology, Psychiatry and Neurosurgery of Chile. La Serena, Chile. October 13, 2000.
- Invited speaker. November 2000. University Clinic, University of Navarra. Pamplona, Spain.
- Invited speaker. November 2000. Ayuntamiento de Murcia, University of Murcia, Spain.
- Invited speaker. March 2001. I International Congress in Neurotoxicity, Pucón, Chile.
- Invited speaker. November 2001. School of Medicine, University of Murcia, Spain.
- Invited speaker, conference. August 24 2002. Associação de Pais e Amigos dos Excepcionais de Sao Paulo (APAE), Sao Paulo, Brazil
- Invited speaker, conference. August 30 2002. Brazilian Federation of Societies of Experimental Biology (FeSBE). Salvador Bahia, Brazil.
- Invited speaker, conference. October 18, 2002. Hôpital de la Salpetriere, Paris, France.
- Invited speaker, conference. October 22nd, 2002. Solvay Pharmaceuticals, Inc. Weesp, Holland.
- Invited speaker, April 2003. II International Congress on Neurotoxicity, La Serena, Chile.
- Invited speaker, September 2003. University of Paris 5, Jussieu. Paris, France.
- Invited speaker, October 2004. NIH, Bethesda, MD, USA.
- Invited speaker, December 2004. USUHS, Bethesda, MD, USA.
- Invited speaker, November 2005. University of Jyväskylä. Jyväskylä, Finland.

PUBLICATIONS

A.- ABSTRACTS, PRESENTATIONS IN MEETINGS:

- 1.- García, P., Caviedes, P., Contreras, M., Codoceo, V. and Caviedes, R. Regulation in the formation of cellular processes in a rat brain cell line in continuous culture. Arch. Biol. y Med. Exp. 13:R-67, 1980.
- 2.- Caviedes, P., Olivares, E. and Cury, M. Electrical activity and calcium fluxes in a cardiac cell line. XXVIII Annual Meeting of the Biology Society of Chile; November 28-30, 1985. Pucón, Chile.
- 3.- Castro, P., Caviedes, P., Contreras, M., Cruces, E. and Romero, M.I. Symptoms of anguish and depression in high school students. XI National Congress of Pediatrics; November 28-30, 1985. Santiago, Chile.
- 4.- Castro, P., Caviedes, P., Contreras, M., Cruces, E., Amat, J. and Romero, M.I. Psychological evaluation of adolescent pregnant women: Symptoms of anguish and depression. XI National Congress of Pediatrics; November 28-30, 1985. Santiago, Chile.
- 5.- León, M., Caviedes, P. and Contreras, M. Control and therapeutical response in the National arterial hypertension control program. V Chilean Public Health Meeting; November 14-16, 1985. Santiago, Chile.
- 6.- Caviedes, P. and Jaimovich, E. Ionic currents in an adult rat cardiac cell line. Arch. Biol. y Med. Exp., 19:R-192, 1986.
- 7.- Caviedes, P. and Rapoport, S.I. Dorsal root ganglion neurons from normal and trisomy 21 human fetal tissue show tetrodotoxin-sensitive and tetrodotoxin-insensitive sodium currents. Soc. Neurosci. Abstr., Vol. 13, 442.5, 1987.
- 8.- Caviedes, P. and Rapoport S.I. Effect of Nerve Growth Factor in the electrical membrane properties of human fetal dorsal root ganglion neurons in culture. Soc. Neurosci. Abstr., Vol.14, Part 2, 331.3, 1988.
- 9.- Caviedes, P. and Rapoport S.I. The role of altered sodium currents in the action potential abnormalities of cultured dorsal root ganglion neurons from trisomy 21 human fetuses. Soc. Neurosci. Abstr., Vol. 15(1), 537, 1989.
- 10.- Caviedes, P. and Vargas, F.F. Cation currents activated by thrombin and bradykinin in microvascular endothelial cells. I Congreso Iberoamericano de Biofísica, Sept. 25 - 28, 1989, Seville, Spain.
- 11.-Caviedes, P. and Vargas, F.F. Agonist and voltage gated ionic currents in human endothelial cells in culture. Gordon Research Conferences, June 10-15, 1990, Plymouth, NH.
- 12.-Caviedes, P., Grant, D. and Vargas, F. F.. Albumin-induced changes in electrical membrane properties of cultured endothelial cells. FASEB Annual Meeting, 1991, Atlanta, GA.

- 13.- Lipicky, R. J., Vargas, F. F., Knudsen, K., Caviedes, P., Grassi, D. and Gill-Kumar, P.. Flecainide effects on voltage dependant sodium and potassium currents. FASEB Annual Meeting, 1991, Atlanta, GA.
- 14.- Tascón, S., Caviedes, P., Hidalgo, J., Jaimovich, E. and Caviedes, R.. Study of differentiated properties in a cloned cell line derived from normal human skeletal muscle. V Annual Meeting of the Society of Cell Biology of Chile, La Leonera, Codegua, Chile, 1991.
- 15.- Caviedes, P., Liberona, J. L., Hidalgo, J. and Jaimovich, E.. Sodium and potassium channels during differentiation of a human skeletal muscle cell line. Arch. Biol. y Med. Exp., 24(2):R-128, 1991.
- 16.- Jaimovich, E., Caviedes, P., Tascón, S., Hidalgo, J. and Caviedes, R.. Ion currents during differentiation of skeletal and cardiac muscle cell lines. Biophysical Journal, 59a, 1992.
- 17.- Fiedler, J., Caviedes, P., Epstein, C.J. and Rapoport, S.I.. Altered cholinergic function in trisomy 16 fetal mouse neurons, an animal model for human Down syndrome. XXXV Annual Meeting of The Biology Society of Chile, Puyehue, Chile, 1992.
- 18.- Berríos, S., Fernández-Donoso, R., Epstein, C.J., Rapoport, S.I. and Caviedes, P. Configuration of tetravalents in mouse spermatocytes. VI Annual Meeting of The Cell Biology Society of Chile, Algarrobo, Chile, 1992.
- 19.- P. Caviedes, J.L. Liberona, R. Caviedes and E. Jaimovich. Expression of ionic channels during the differentiation of muscle cell lines in culture. I Congreso Iberoamericano de Biofísica, October 3 - 7, 1993, Puebla, Mexico.
- 20.- Berríos, S., Fernández Donoso, R., Martínez, S. and Caviedes, P.. Number and nuclear distribution of multivalent telomeres in meiosis. Annual Meeting of the Genetics Society of Chile, 1993.
- 21.- Caviedes, P., Olivares, E., Salas, K., Caviedes, R. and Jaimovich, E.. Calcium fluxes, ionic currents and dihydropyridine receptors in a new, immortal cell line of rat cardiac muscle. XXXVI Annual Meeting of the Biology Society of Chile, Puyehue, Chile, November 24-27, 1993.
- 22.- Caviedes, P., Caviedes, R., Liberona, J.L. and Jaimovich, E.. Ion channels and receptors in skeletal muscle cell lines from Duchenne muscular dystrophy patients. Biophys. J., 66(2) (1994), A90.
- 23.- Jaimovich, E., Caviedes, P., Liberona, J.L., Hidalgo, J. and Caviedes, R.. Developmental expression of ion channels in cultured muscle cell lines. Symposium "Ion Channel Pharmacology". Alicante, Spain, 1994.
- 24.- Fiedler, J.L., Rapoport, S.I., Caviedes, R. y Caviedes, P.. Cholinergic function in the Central Nervous System of trisomy 16 fetal mice, an animal model for human trisomy 21 (Down syndrome). XIV Latinamerican Congress of Pharmacology, November 20 - 24, 1994. Santiago, Chile.
- 25.- Caviedes, P., Vargas, F.F. and Grant, D.S.. Electrophysiological properties of endothelial cells in culture: Alterations related to changes in the external concentration of albumin.

Symposium "Functions of vascular endothelium". XIV Latinamerican Congress of Pharmacology, November 20 - 24, 1994. Santiago, Chile.

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E.- PATENTS

- 1.- Caviedes, P., Caviedes, R., Freeman, TB., Sanberg, P.R. y Cameron D.F. Proliferated Cell Lines and Uses Thereof. International Publication number WO 03/065999 A2. Filed Feb. 8th, 2002, publication date: August 14th, 2003. World Intellectual Property Organization, International Bureau.
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THESIS DIRECTED

i) Ongoing:

- Esteban González. Thesis director, PhD program in Medical Sciences Faculty of Medicine, Univ. of Chile. Stem cell differentiation to cardiomyocytes: Application to cell transplant therapy in myocardial infarction.
- Patricio Cabané Toledo. Thesis director, PhD program in Medical Sciences Faculty of Medicine, Univ. of Chile. Optimization of culture conditions for parathyroid cells: Cell transplant therapy in Hypoparathyroidism.
- Jura Mikkola. Thesis director, MSc in Neuroscience. University of Jyväskylä, Finland. Effect of Sod1 and Sod1/App knockdown in cholinergic dysfunction of a cell line derived from the cerebral cortex of a trisomy 16 mouse, an animal model of Down syndrome.
- Adriana Armijo. Thesis director, MSc in Neuroscience, Faculty of Medicine, Univ. of Chile. Characterization of a cell line derived from the motor cortex of an adult rat as a model for cell transplant therapy in cerebral ischemia.
- Paola Fernández Olivares. Thesis director, Bach. In Biochemistry, Univ. Católica de Valparaíso. Effect of Slc5a3 (SMIT1, mioinositol transporter) knockdown in glutamatergic dysfunction of a cell line derived from the cerebral cortex of a trisomy 16 mouse, an animal model of Down syndrome.
- Ignacio Díaz Franulic. Thesis director, Bach. In Biochemistry, Univ. of Chile. Effect of Slc5a3 (SMIT1, mioinositol transporter) knockdown in cholinergic dysfunction of a cell line derived from the cerebral cortex of a trisomy 16 mouse, an animal model of Down syndrome.

ii) Finished:

- Irmgard Paris. Thesis codirector, PhD program in Biomedical Sciences, Faculty of Medicine, Univ. of Chile. Studies on neurotoxic effects of copper in dopaminergic cell lines. Exam: June 30, 2006.
- Patricia Opazo. Undergraduate thesis, Biochemistry, Univ. Católica de Valparaíso. Effect of APP knockdown on cholinergic dysfunction in a neuronal cell lines derived from the cerebral cortex of a trisomy 16 mouse, an animal model for Down syndrome. Exam: December 14, 2005.

- Guillermo Rojas. Undergraduate thesis, Biochemistry, Univ. Católica de Valparaíso. Effect of APP knockdown on glutamatergic dysfunction in a neuronal cell lines derived from the cerebral cortex of a trisomy 16 mouse, an animal model for Down syndrome. Exam: January 4, 2006.
- Robert Flint, Codirector, Pre PhD thesis work. University of Utrecht, Holland. MAP kinase dependent mechanisms in cell models of Parkinson's disease.
- Andrea García Pietrobon. Co director, undergraduate thesis de pregrado, Chemistry & Pharmacy, Fac. de Ciencias Químicas y Farmacéuticas, Universidad de Chile. Cell toxicity by HTLV virus in neuronal cell lines.
- Christian Arriagada A., Ms.C. Program in Biological Sciences, Faculty of Medicine, Univ. of Chile
- Darío Sepúlveda, Undergraduate Thesis in Chemical Engineering, Faculty of Physycal & Mathematical Sciences, University of Chile. Optimization of culture techniques in dopaminergic cell lines: Applications in the isolation of cell products and cell transplant December 2001
- Lori B. Bennett. Undergraduate thesis, Marlboro College, Vermont. Cholinergic function in cell lines derived from the spinal cord and dorsal root ganglia of normal and trisomy 16 mice. Defense: May 10th, 2001. Grade: A.
- Alexis Olivares. Co director, thesis for the degree of Chemistry & Pharmacy, Faculty of Chemistry & Pharmacy, Univ. of Valparaíso Title::Characterization of neurotransmitter receptors in cell lines derived from the spjnal cord of normal and trisomy 16 mice. November 2000.
- José Cortés. Co director, thesis for the degree of Chemistry & Pharmacy, Faculty of Chemistry & Pharmacy, Univ. of Valparaíso Title: Intracellular Ca^{2+} signals in hippocampal cell lines from normal and trisomy 16 mice. December, 1999
- José Martín. Director, thesis for the degree of Chemistry & Pharmacy, Faculty of Chemistry & Pharmacy, Univ. of Valparaíso Title: Characterization of the cholinergic function of neuronal cell lines derived from the cerebral cortex of normal and trisomy 16 mice. January, 2000.
- Christian Arriagada. Director, degree of Laboratory Technician, Faculty of Medicine, Univ. of Chile. Thesis title: Morphological characterization and cloning of neuronal cell lines from normal and trisomy 16 mice, an animal model of human Down syndrome.. March, 1999.
- Maribel Rodríguez.. Co director, degree in Chemistry & Pharmacy, , Faculty of Chemistry & Pharmacy, Univ. of Valparaíso Thesis title: Intracellular calcium movements in neuronal cell lines from the cerebral cortex of normal and trisomy 16 mice. March, 1997.
- Paola Reyes. Co director, degree in Chemistry & Pharmacy, Faculty of Chemistry & Pharmacy, Univ. of Valparaíso Thesis title: Immunohystochemical and pharmaceutical characterization of a cerebellar neuronal cell line. March, 1996.

MOST RELEVANT CURRENT COLLABORATIONS:

- Dr. Ana María Cárdenas, Neuroscience Center, Univ. of Valparaíso. Study of Ca^{2+} signals (intracellular concentration) in response to neurotransmitters.
- Dr. Juan Segura-Aguilar, ICBM, Faculty of Medicine, Univ. of Chile. Neurodegenerative mechanisms related to Parkinson's disease.
- Dr. Enrique Jaimovich, ICBM, Faculty of Medicine, Univ. of Chile. Characterization of muscle cell lines.
- Dr. Juan Asenjo, Biotechnology Center, Faculty of Engineering, Univ. of Chile. Biomolecules, cell line products.
- Dr. Stanley Rapoport, Lab Neurosciences, National Institute on Aging, NIH. Bethesda, MD, USA.. Establishment and characterization of neuronal cell lines from normal and trisomy 16 mice.
- Dr. David Allen, Texas Tech University HSC, Amarillo, TX. Study of cholinergic function
- Drs. Takeshi Shimahara and Jordi Molgó, CNRS, Gif-sur-Yvette, France.. Ca^{2+} signal imaging (funded by CNRS/Conicyt exchange program)

- Dr. Rita Raisman-Vozari, INSERM, France. Neuroplasticity of substantia nigra cell line in response to neuromodulators.
- Dr. Thomas Freeman, Univ, of South Florida, Tampa, FL. Neuronal cell lines in cell transplant therapy for neurodegenerative diseases.
- Dr. Andreas Seyfang. Univ, of South Florida, Tampa, FL Purification and Characterization of a Transformation Factor Secreted by a Rat Thyroid Tumor Cell Line
- Dr. Sergio T. Ferreira. Univ. of Rio de Janeiro, Brazil. Amyloid neurotoxicity in trisomy 16 neuronal cell lines.



EXHIBIT B



Arriagada, C., Salazar, J., Shimahara, T., Caviedes, R. and Caviedes, P. An immortalized neuronal cell line derived from the substantia nigra of an adult rat: Application to cell transplant therapy. In: "Parkinson's Disease", E. Ronken & G. van Scharrenburg, editors. *IOS Press, Amsterdam, Netherlands* (2002), ISBN 1 58603 207 0, pp. 120-132.

AN IMMORTALIZED NEURONAL CELL LINE DERIVED FROM THE SUBSTANTIA NIGRA OF AN ADULT RAT: APPLICATION TO CELL TRANSPLANT THERAPY.



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Immortalized cell lines offer great advantages over other *in vitro* preparations. Indeed, cell lines can provide large amounts of genetically homogeneous tissue, and are readily accessible and easy to manipulate. However, cell lines must deal with problems such as lack of stability, which may result in loss of differentiated properties and death. In our lab, we have successfully produced immortalized cells lines of various tissues from adult mammalian donors (rat, mouse, bovine, human), using an original transformation protocol. Briefly, primary cultures are kept in the presence of media conditioned by the UCHT1 rat thyroid tumoral cell line, which induces transformation after variable periods of time. In this presentation, we discuss the characteristics of the neuronal cell RCSN, derived from the *substantia nigra* of an adult rat. By immunohistochemistry, the cell line shows positive neuronal markers (NSE, synaptophysin, MAP-2, neurofilament) and lack glial traits (GFAP, S100). RCSN cells possess catecholaminergic traits: presence of TH, melanin and DAT. This cell line also line exhibits intracellular Ca^{2+} increments in response to excitatory neurotransmitter agonists. Finally, using stereotaxic surgery, we implanted suspensions of this cell line (500.000 cells in 4 μL) in the striatum of rats previously lesioned in the nigrostriatal pathway with 6 OH dopamine. The implanted rats show a steady decrease in rotations, leveling off at 75% of the initial rotation values after 16 weeks post implant. Sham operated rats, where only the vehicle was implanted, show no improvement. The results suggest that the RCSN cell line appears as a model for the study of cell mechanisms related to Parkinson's like neurodegeneration. The effect of the implanted RCSN cell line in the improvement of the rotational behavior in 6 OH Dopamine lesioned rats is encouraging in our quest to establish a human substantia nigra cell line with the UCHT1 protocol, and generate a model that could be applied towards cell transplant therapy in humans.

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Parkinson's disease is a neurodegenerative disorder affecting an estimated one million patients in the United States alone (1), and it is determined by the degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SN) which project to the striatum. The cardinal features of Parkinson's disease are: **tremor**, mainly at rest; **muscular rigidity**, which leads to difficulties in walking, writing, speaking and masking of facial expression; **bradykinesia**, a slowness in initiating and executing movements; and **stooped posture and instability** (2). In later stages of the disease, patients undergo cognitive deterioration. The degeneration of the SN determines an imbalance in the regulation of the circuitry of the basal ganglia, whose GABAergic output to the thalamus is increased, therefore determining an inhibition of this projection nucleus (3). Hence, messages relayed from the thalamus to the cortex, especially those coding for voluntary movement, are slowed down.

Many strategies are being pursued to develop new therapies for Parkinsonian patients, oriented to prevention of the damage of the nigrostriatal system and to the replacement of lost neurons. These techniques range from the use of dopaminotrophic factors (4) and viral vectors (5), to the transplantation of primary xenogeneic tissue (6). Cell transplantation with fetal tissue (26), xenograft from porcine tissue (7,8) and immortalized cell lines (9,10,11) appear as clinically promising experimental treatments in late stage Parkinson's disease, where tissue replacement is essential. Indeed, considering that Parkinson's patients develop symptoms when approximately 50-70% of the dopaminergic neurons in the SN are lost (3), the development of cell transplant models appears as a much needed step in the cure for the disease. Further, the discrete anatomical localization of the degenerating tissue make it a likely candidate for cell transplant therapy with minimally invasive stereotaxic procedures.

More than two hundred patients have received transplants worldwide (12). Clinical improvement has been confirmed by functional studies using positron emission tomography of striatal fluorodopa (F-DOPA) uptake after transplantation (12,13) and was correlated to good graft survival and innervation of the host striatum in postmortem studies of transplanted patients (1). However, and in spite of these promising findings, neural transplantation remains a controversial procedure, facing ethical dilemmas in procurement of tissue (human, human fetal), and in obtaining adequate amounts of cells. The development of neuronal, dopaminergic cell lines from the SN would greatly contribute to overcome these difficulties, and would provide a limitless supply of cells that could be easily manipulated and modulated *in vitro* prior to transplantation. At present, successful attempts to establish

such lines have been made in rat SN, using SV40 virus transfection (9,10,11). However, cell lines so established tend to exhibit problems in their stability and/or viability (14). Our group has successfully established a continuously growing cell line from the SN of an adult Fisher 344 rat (15,16), named RCSN-3, using an original protocol developed in our laboratory which has yielded continuously growing cell lines from mammalian tissue of diverse origins that stably retain differentiated traits (16-21). In the present work, we show that the RCSN-3 cell line presents neuronal markers by immunohistochemistry and lack glial traits. We also transplanted suspensions of this cell line in the striatum of 6 OH dopamine lesioned rats, achieving up to 75% recovery in the rotational behavior of the animals.

Part of this work has been presented in abstract form (22).

MATERIAL AND METHODS

Cell culture

a) Establishment of cell lines. The RCSN-3 cell line was derived from the substantia nigra of 4 month old normal Fisher 344 rat. The cell material used to establish primary cultures was then transformed to a permanent cell line by exposing them to media conditioned by UCHT1 cells (Figure 1), a process that induces transformation in cell cultures (16-22). For standard culture conditions, the cells were kept in feeding medium consisting of DMEM/ Ham F12 nutrient mixture (1:1) (Sigma Chemical Co., Saint Louis, MO, USA) modified to contain 6 g/l glucose, 10 % bovine serum, 2.5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma) supplemented with 10% (v/v) with UCHT1 conditioned medium. The cultures were maintained in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO₂ and were monitored routinely for the appearance of transformation foci or morphological changes. After 10 weeks in culture, transformation foci were evident. The cultures were expanded and part were cryopreserved in liquid nitrogen. The cell line was cloned by dilutional culturing, giving rise to the clonal line RCSN-3. Cells are passaged at confluence with trypsinization (1% trypsin, Gibco, Grand Island, NY, USA).

b) Culture of cell lines. For standard growth conditions, RCSN-3 CNh cells were cultured in feeding medium. Media was renewed completely twice a week.

For differentiation, the cell were kept in a media consisten of DMEM/Ham F12 nutrient mixture, supplemented with 2% adult bovine serum and 1% (v/v) of N3 supplement as previously described (18) and 1% (v/v) Site+3 supplement (Sigma). Cell were allowed to differentiate for 1 week.

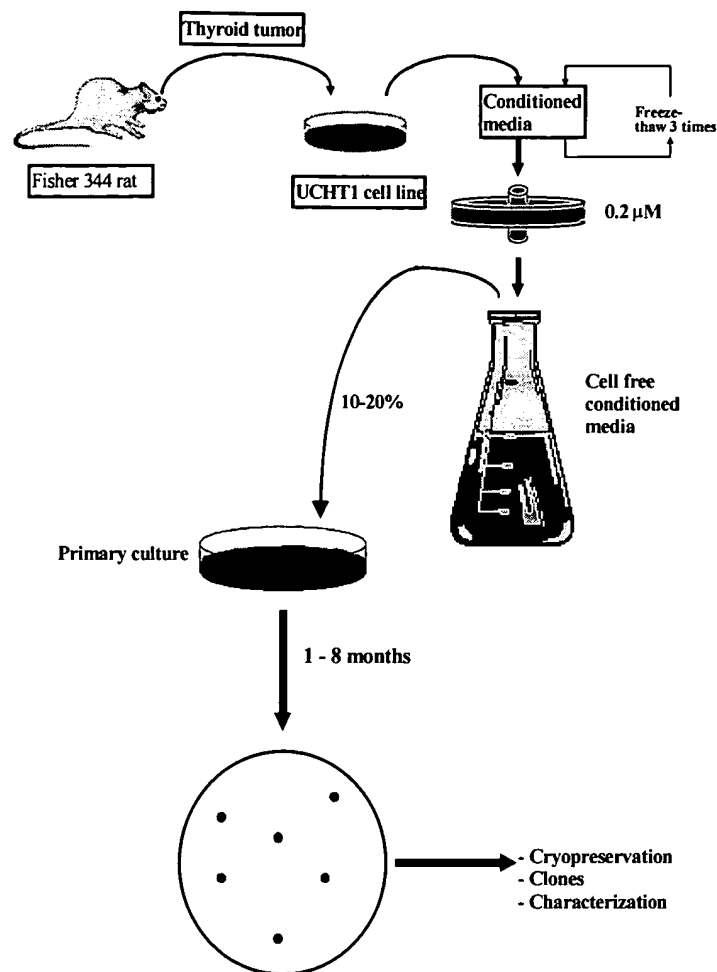


Figure 1. UCHT1 transformation protocol. Media conditioned by the rat thyroid UCHT1 cell line for 48 hrs. is freeze-thawed 3 times in the absence of cryopreservants. The media is filtered through 0.2 µm filters to yield a cell free conditioned media. Primary cultures of mammalian origin are kept in the presence of 10-20% (v/v) for the time range noted. Successful transformation is assessed by the generation of transformation foci in the culture.

Morphology

Cells were fixed in formaldehyde 4% in phosphate buffer pH 7.4.

a) **Citology.** Cytochemical reactions included: Hematoxylin – eosin staining, ferrous ion capture to demonstrate the presence of melanin in the form of neuromelanin, paraformaldehyde-glyoxylate staining to demonstrate the presence of catecholamines

b) **Immunohistochemistry.** Fixed cells were permeabilized in an ascending/descending alcohol battery ranging from 50 to 96%. The blocking reaction was carried out using BSA 1% in phosphate buffer. The antibodies utilized here were the next: 1.- neuronal markers: NSE (pre-diluted, Biogenex), Synaptophysin (pre-diluted, Biogenex) and MAP-2 (1:1000, Sigma) 2.- glial markers: GFAP (pre-diluted, Biogenex) and S-100 (pre-diluted, Biogenex) 3.- Functional markers: TH (1:1000 – 1:1500, Sigma). The incubation with the primary

antibodies was carried out overnight and an ABC detection kit (Biogenex) was used to develop the reaction and utilizing DAB as chromogen. Specific primary antibodies, fluorescein labeled secondary antibodies and tetanus toxin (kind gifts, Dr. Lautaro Pérez, ICBM) were used to evaluate the presence of neurofilament 200 kD and tetanus toxin receptor.

Intracellular Ca^{2+} measurements

For intracellular Ca^{2+} measurements, the cells were plated onto 35 mm culture dishes. The variations of intracellular Ca^{2+} were assessed by Ca^{2+} imaging techniques using Fluo-3. The cells were incubated at 37°C for 40-60 min with the indicator. The dishes were visualized in an Olympus BH2 microscope equipped with epifluorescence (halogen lamp). The microscope was connected to a Cooled Extended Isis digital camera (Photonic Science Ltd, Robertsbridge, UK) connected to a dedicated PC equipped with a Axon Digidata 2000 digitizing board (Axon Instruments, Foster City, CA). Images were acquired at 12 bit resolution and 1 Hz using a customized software Axon Imaging Workbench 2.1.80 (Axon), and stored in the computer hard disk for later analysis.

The compositions of the normal extracellular solutions were (in mM): 135 or 145 NaCl, 5 KCl, 2 $MgCl_2$, 1.5 or 2.5 $CaCl_2$, 10 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-NaOH, 10 Dextrose (pH=7.4).

Surgical procedures, behavioral testing.

4 adult male Fisher 344 rats (200–250 g) were lesioned by unilateral injection of 6-hydroxydopamine bromide at two sites along the medial forebrain bundle. Assessment of apomorphine induced rotational behavior (i.p injection of 5 mg apomorphine per kg body weight, National Health Service, Chile) was carried out visually twice, once per week, before transplantation. Only rats with more than 160 rotations every 30 min were utilized and three times after transplantation (days 30, 55 and 80). For transplant, confluent cultures were washed in PBS and dissociated with 1% trypsin. 500.000 cells in a volume of 4 μ L were implanted through blunt Hamilton syringe and deposited at AP +1.0 mm, ML -2.5 mm and V -4.7 mm (coordinates relative to bregma), toothbar set at -2.5. Rotational behaviour was assessed visually every two weeks after transplantation.

RESULTS

The RCSN-3 cell line grows on monolayers, with a doubling time of 52 hrs, a plating efficiency of 21% and a saturation density of 410.000 cells/cm², when kept in feeding medium. Figure 2 shows that undifferentiated RCSN cells tend to exhibit a epithelial like morphology, with short or no processes and a more acidophylic cytoplasm. After differentiation, cell proliferation is greatly reduced, and RCSN cells develop processes and establish contact with neighboring cells. The presence of melanin was evidenced with the ferrous ion capture technique, demonstrating a homogeneous distribution of the pigment in the cytoplasm, with faint labelling in undifferentiated stages and a substantial increase upon differentiation.

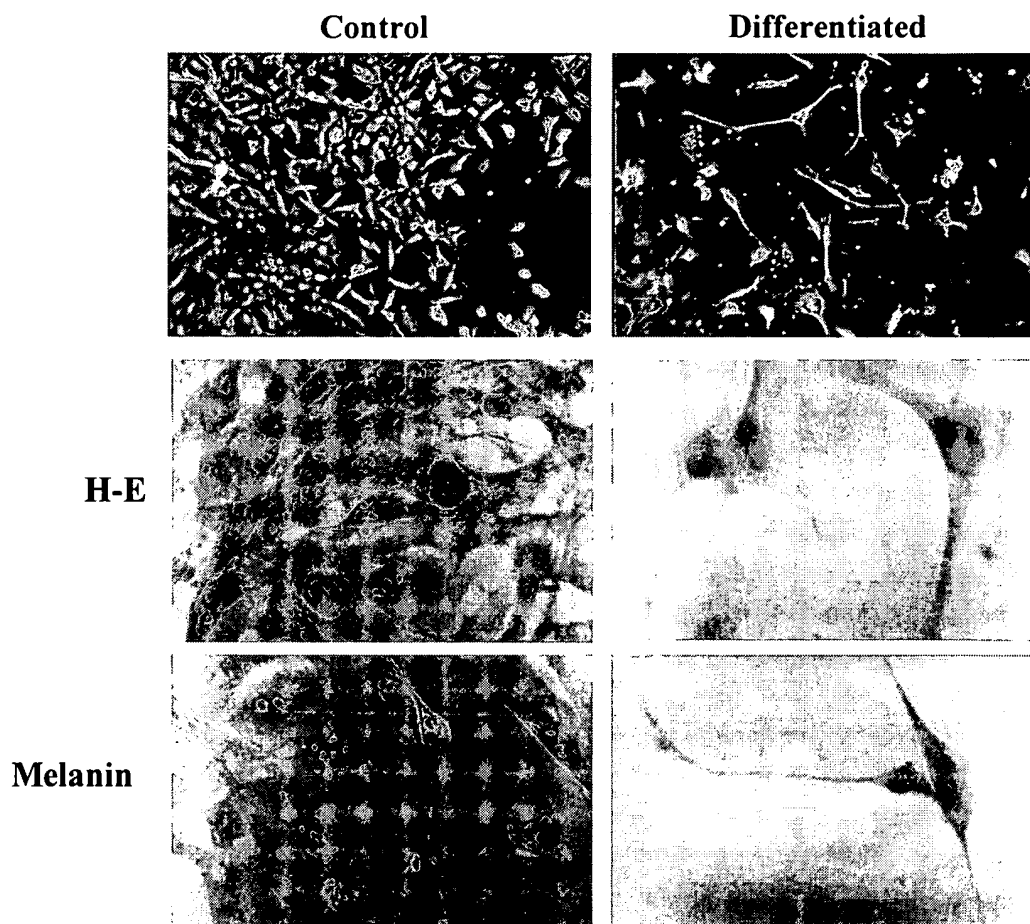


Figure 2. The top panels represent phase contrast microscopy images. Note the decrease in cell number and the increased length and number of dendrites. H-E: Hematoxylin-eosin.

Immunohistochemical characterization demonstrates that RCSN cells express neuronal traits, evidenced by the positive immunolabelling for NSE, synaptophysin and MAP-2. NSE and synaptophysin show a finely granular pattern, evenly distributed in the cytoplasm (Figure 3). Synaptophysin is specially intense at the zone of cell-cell interaction. MAP-2 shows a fibrillary pattern of labelling, surrounding vacuole-like cytoplasmic structures. Microfilament 200 kD labels differentiated cells homogeneously, and tetanus toxin is present in the cell membrane in a patch-like distribution.

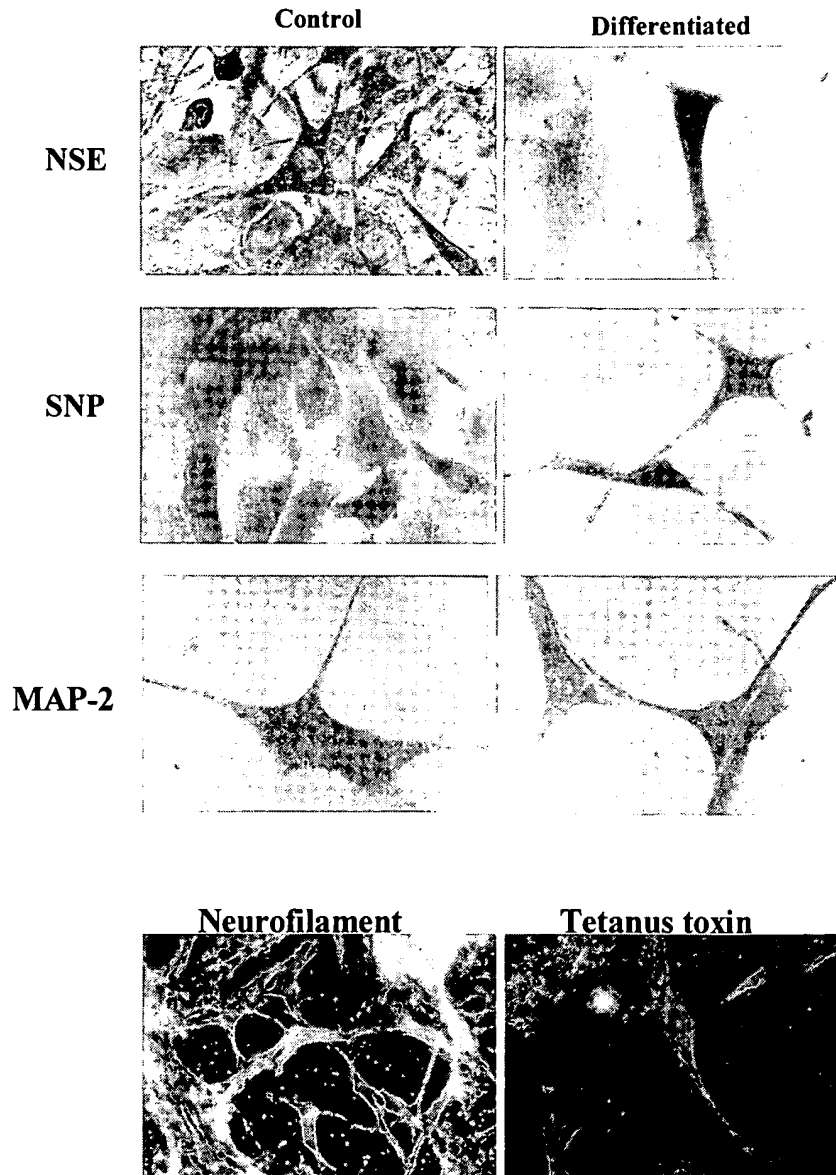


Figure 3. Immunohistochemistry for neuronal markers. NSE: Neuron specific enolase. SNP: Synaptophysin. MAP-2: microtubular associated protein-2. The bottom panels represent images taken under epifluorescence microscopy conditions, taken in differentiated cells.

Paraformaldehyde-glyoxylate

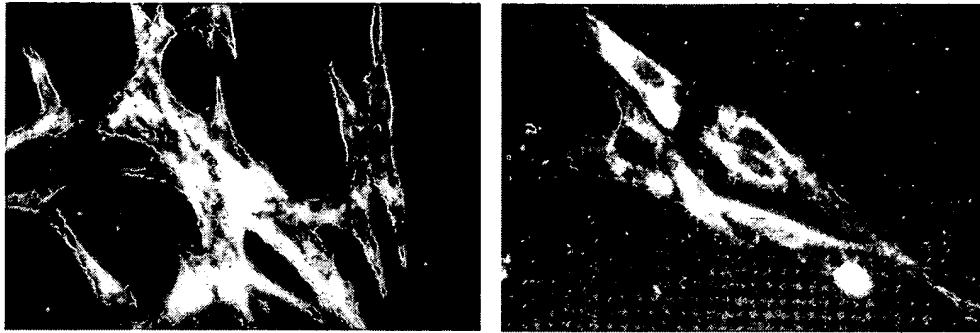


Figure 4. The top panels represent immunohistochemistry for tyrosine hydroxylase (TH) in RCSN cells, under control and differentiated conditions. The bottom panels Micrograph of RCSN cells using the ferrous ion capture technique, where fluorescent areas represent catecholamine deposits.

Functional neuronal markers are shown in figure 4, which presents immunohistochemical staining for tyrosine hydroxylase. The labeling is slightly less intense in non-differentiated cells, and the label is distributed in the entire cytoplasm following a granular pattern. The presence of catecholamines is also clear from the micrographs presented in figure 4, with a cytoplasmic distribution. Glial markers GFAP and S100 were negative in both control and differentiated conditions (data not shown).

When differentiated, up to 40% of fluo-3 loaded RCSN cells respond with intense increase in intracellular Ca^{2+} when stimulated externally with 200 μM glutamate, and even more intensely when using simultaneous depolarizing conditions (70 mM K^+), a situation depicted in figure 5. Of 16 cells explored, the Ca^{2+} signal peaks after 1 sec. of stimulation, and returns to basal level between 30-40 sec. after the peak.

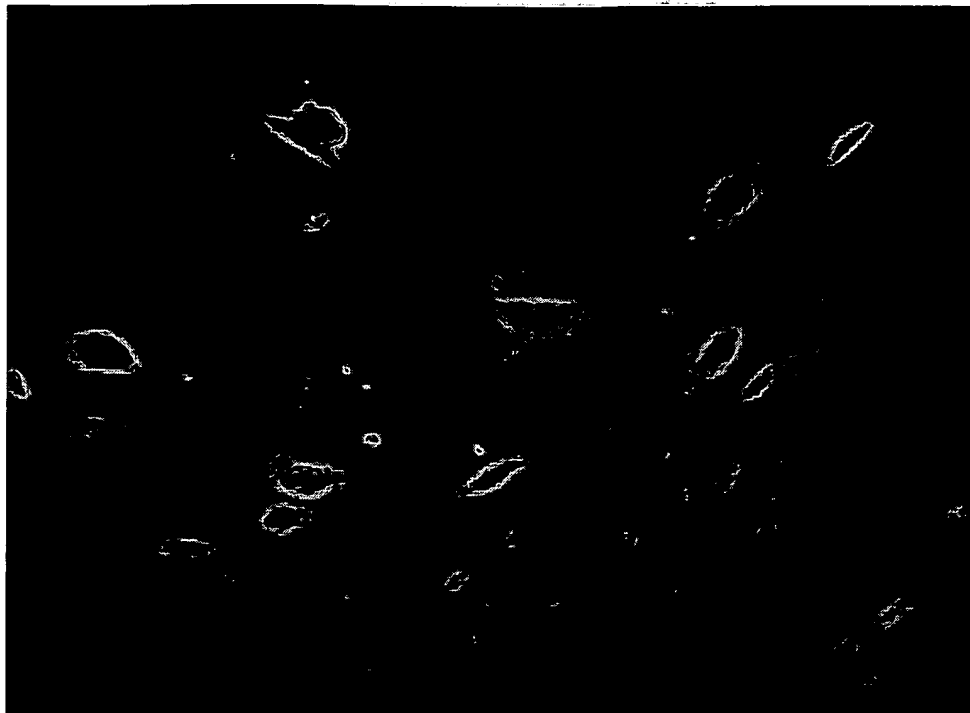


Figure 5. Ca^{2+} signals in fluo-3 loaded RCSN lines. The image shows cells 3 sec after being stimulated with the addition of 200 μM glutamate, and even more intensely when using simultaneous depolarizing conditions (70 mM K^+). Fluorescence intensity is depicted in a pseudo color scale, which in ascending order is black – blue – green – yellow – orange – red.

Finally, we transplanted suspensions of RCSN cells in the striatum of rats with 6OH dopamine- induced lesions of the substantia nigra. Figure 6 shows typical patterns in the evolution of the rotational behaviour after transplantation, characterized by either a smooth , decreasing exponential type curve which level off after 12 weeks at approximately 25% of the initial rotation rate. Another pattern involves a greater drop in rotations 2 weeks after transplantation, followed by an increase and later a sustained decrease in the rate of rotation to again plateau after 12 weeks. At 16 weeks, the rats were sacrificed, and section of the striatu were taken and immunohistochemically stained with TH and DOPA decarboxilase antibodies. As shown in figure 5, cells staining positively for both markers are present in the striatum, showing intense labeling and neurite formation.

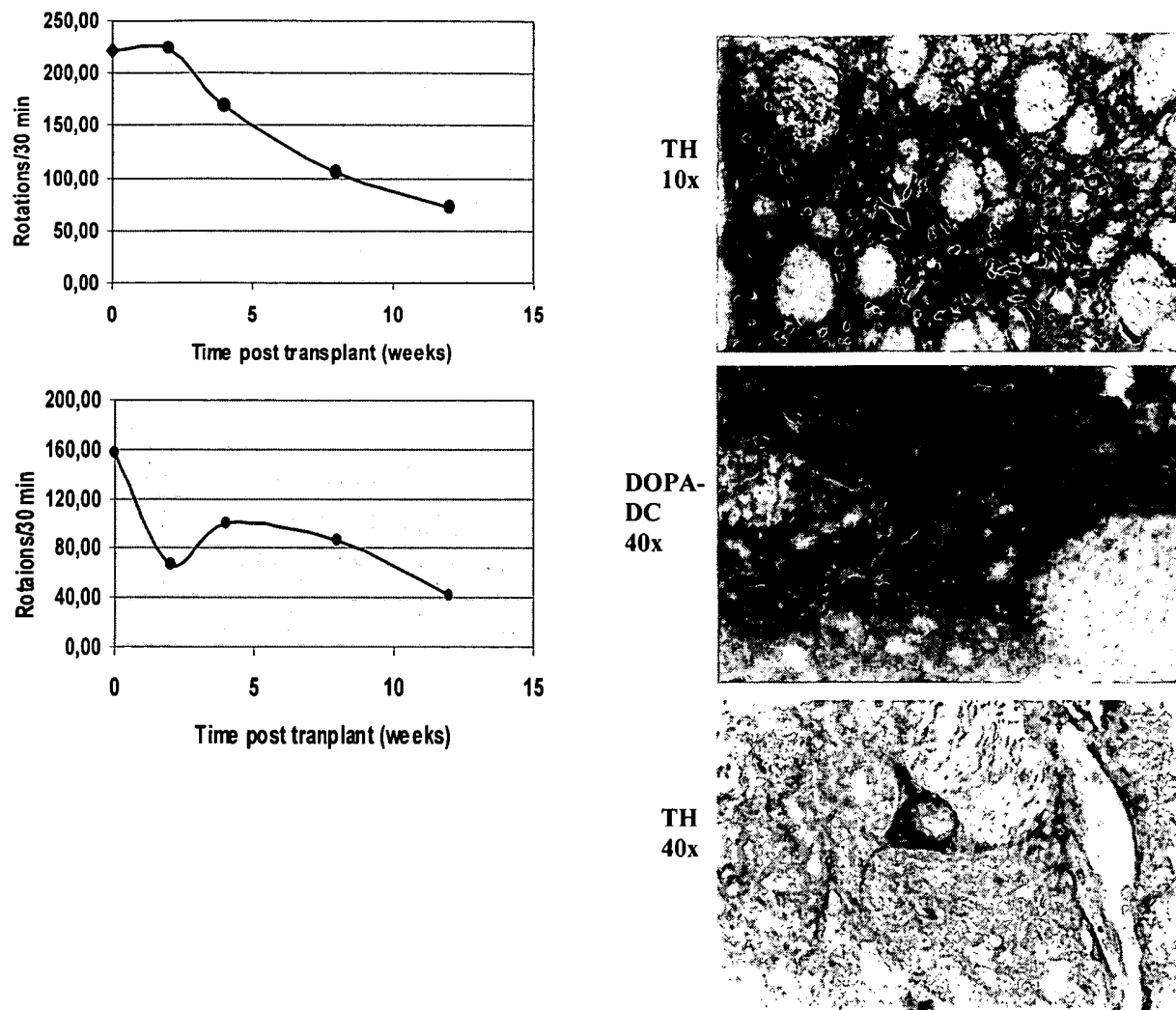


Figure 6. Transplant of RCSN-3 cells in the striatum of 6OH dopamine lesioned rats. The graphs at the left represent the decrease in the rate of rotation after transplant. The microphotographs on the right represent tyrosine hydroxylase (TH) and DOPA decarboxylase (DOPA-DC) immunostaining in striatal sections of two rats sacrificed 16 weeks after transplantation.

DISCUSSION

The use of immortalized cell lines to study basic phenomena at the cellular and molecular levels is highly desirable. Ideally, such cell models should derive from the differentiated tissue, and retain the properties of the originating tissue *in vitro*. However, current transformation protocols have yet to overcome problems involving viability and/or stability (14). Stem cells are a promising possibility, but finding the adequate *in vitro* conditions to attain the state of differentiation desired in such cells can be cumbersome and costly. Our UCHT1 transformation protocol seems to overcome these drawbacks to the extent that all our cell lines so generated retain differentiated properties for extended periods of time (16-21). Further, we have even immortalized pathological tissue, such as the cerebral cortex of a mouse bearing an extra copy of chromosome 16 (17,18), an animal model of

Down's syndrome, which retains the same neurotransmitter dysfunction previously observed in primary cultures of the same origin(23). The present study shows that the RCSN-3 clonal cell line retains general properties of neuronal tissue, and possesses specific characteristics of the SN, such as the presence of tyrosine hydroxylase, DOPA decarboxylase and catecholamines. Previous studies have shown that this cell line exhibits neurotoxicity in response to the external application of salsolinol(22), a dopamine-derived isoquinoline which reportedly acts as an endogenous dopaminergic neurotoxin, inducing selective neuronal cell death and eliciting symptoms almost identical to idiopathic Parkinson's disease in humans (24). Further, salsolinol has been reported to induce behavioral changes similar to those observed in Parkinson's disease (25), making it an interesting candidate for the study of dopamine induced toxicity. If we also consider that RCSN-3 cells respond to glutamate with increases in intracellular Ca^{2+} , the cell line appears as a likely candidate for the study of excitotoxic mechanisms involved in neurodegeneration, as well as more specific mechanisms related to dopamine derived neurotoxicity.

However, a very exciting application arises from our present study. The RCSN-3 cell line appears as a model for cell transplant therapy, inducing a sustained and progressive reduction in the rotational behavior of 6 OH dopamine lesioned rats. Interestingly, no previous *in vitro* differentiation was utilized in our transplantation experiments, which may prove to be a practical asset, as the cells either have enough dopaminergic function at the time of the inoculation, or the *in vivo* microenvironment in the striatum may be enough to sustain or induce a differentiated phenotype in the RCSN-3 line.

Cell grafting in Parkinsonian models has been the subject of intense study (26). Procedures have involved the use of primary mesencephalic cells (12,27), genetically modified cells that do not differentiate into neurons in the host striatum (28), and dopaminergic neurons derived from precursors expanded *in vitro* (1). The latter may reflect the situation of RCSN-3 cells more closely, as it offers many of the advantages of expanded precursors. Indeed, the expansion of cells in culture followed by a differentiation period or genetic manipulation may reduce the amount of tissue needed and provide optimal cell material. However, it must be pointed out that non-expanded neuronal precursors have shown differentiation *in vivo* when transplanted(29-32), *in vitro* expanded cells have been less efficient in developing into neurons once transplanted (33,34). It appears that in the latter case a differentiation period in culture, rather than just an expansion of the cells, is necessary(1). Previous work with primary fetal mesencephalic tissue suggests that

behavioral recovery be directly correlated to the number of surviving dopaminergic neurons in the host striatum(35) . We cannot adequately address this issue in our study, since we have used a reduced number of animals, although the survival rate reported in successful grafts of dopaminergic neurons is rather low (3-5%)(12). From the study in the striatal sections in our transplanted animals, our RCSN-3 cell line appears well in these figures, with the added advantage of not requiring prior differentiation in vitro.

Our present study presents the RCSN-3 cell line as a model to study Parkinson's related mechanisms at the cellular level, and presents the UCHT1 protocol as a method for generating limitless cellular material for cell transplant. It is tempting to speculate that this cell line may also be successful in transplantation in higher order of mammal, such as primates. Indeed, recent reports of successful xenografting of porcine cells in primates (36,37) is encouraging, and we therefore intend to transplant the RCSN-3 cells in MPTP primates shortly. Undoubtedly, the success of xenografting would expand our potential sources of tissue, and provide more opportunities of generating adequate immortalized cell lines to propose for transplantation in humans.

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EXHIBIT C

Clustered RCSN cells, 6- OH dopamine rats

